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1 **Identification of host trafficking genes required for HIV-1**
2 **virological synapse formation in dendritic cells**

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21 ABSTRACT

22 Dendritic cells (DC) are one of the earliest targets of HIV-1 infection acting as a
23 ‘Trojan Horse’, concealing the virus from the innate immune system and delivering it
24 to T-cells via virological synapses (VS). To explicate how the virus is trafficked
25 through the cell to the VS and evades degradation, a high-throughput siRNA screen
26 targeting membrane trafficking proteins was performed in monocyte-derived
27 dendritic cells (MDDC). We identified several proteins including BIN-1 and RAB7L1
28 that share common roles in transport from endosomal compartments. Depletion of
29 target proteins resulted in an accumulation of virus in intracellular compartments
30 and significantly reduced viral *trans*-infection via the VS. By targeting endocytic
31 trafficking and retromer recycling to the plasma membrane, we were able to reduce
32 the virus’s ability to accumulate at budding microdomains and the VS. Thus, we
33 identify key genes involved in a pathway within DC that is exploited by HIV-1 to
34 traffic to the VS.

35

36 IMPORTANCE

37 The lentivirus Human Immunodeficiency Virus (HIV) targets and destroys CD4+ T-
38 cells, leaving the host vulnerable to life-threatening opportunistic infections
39 associated with Acquired Immunodeficiency Syndrome (AIDS). Dendritic cells form a
40 Virological synapse (VS) with CD4+ T-cells, enabling the efficient transfer of virus
41 between the two cells. We have identified cellular factors that are critical in the
42 induction of the VS. We show that ARF1, BIN1, RAB7L1 and RAB8A are important
43 regulators of HIV-1 trafficking to the VS and therefore infection of CD4+ T-cells. We

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44 found these cellular factors to be essential for endosomal protein trafficking and
45 formation of the VS, depletion of target proteins prevented virus trafficking to the
46 plasma membrane by retaining virus in intracellular vesicles. Identification of key
47 regulators in HIV-1 trans-infection between DC and CD4+ T-cells has the potential for
48 development of targeted therapy to reduce trans-infection of HIV-1 *in vivo*.

49

50 INTRODUCTION

51 Dendritic cells (DC) are key antigen-presenting cells that provide an
52 important link between innate and adaptive immune systems, activating T-cells
53 (reviewed in (1, 2)). Although HIV-1 is able to replicate in DC, the process is
54 inefficient and produces low levels of infectious virus (3-8). However, DCs are able to
55 transfer intact viral particles to target T-cells via a virological synapse (VS) by a
56 process termed *trans*-infection (9), contributing to the spread of infection *in vivo* (10,
57 11).

58 HIV-1 *trans*-infection has been shown to depend on the ability of the virus to
59 'surf' along the surface of the DC via actin rich dendrites, to promote *trans*-infection
60 (12-14). Several studies conducted in macrophages and DC located virus
61 sequestered into plasma membrane invaginated compartments from which viral
62 particles are released at the VS (15-18). These compartments are thought to be
63 surface accessible (15), however there is evidence of a population becoming isolated
64 from the cell surface (16). It is established in macrophages and DC that these surface
65 accessible compartments may have complex morphologies that require membrane

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66 trafficking regulation, such as the virus containing compartments found in
67 macrophages (17).

68 In contrast, *cis*-infection of DC is limited by the host restriction factor
69 SAMHD1, a dinucleotide triphosphate (dNTP) hydrolase that blocks reverse
70 transcription of viral DNA (19-24). In addition, viral cytosolic DNA is sensed by cGAS,
71 a GMP-AMP synthase that induces an interferon type I response in DC (25-27)
72 restricting productive viral replication.

73 It has been previously reported that HIV-1 virus enters the cell through the
74 endolysosomal pathway with evidence supporting roles for clathrin-mediated
75 endocytosis (4, 28, 29), receptor-mediated endocytosis (30, 31) and
76 macropinocytosis (32). However, at later time points virus accumulates in virus
77 containing compartments, rich in tetraspanins such as CD81 that are continuous with
78 the plasma membrane (4, 15, 17). More recent studies identified the importance of
79 tetraspanin 7 (TSPAN7) and dynamin 2 (DNM2) in maintaining viral particles on
80 dendrites and promoting efficient viral transfer. Disruption of these targets led to
81 sequestration of virus in intracellular vesicles and a reduction in viral transfer (13).

82 To elucidate the role of membrane trafficking in the capture and trafficking of
83 the virus through DC to the VS, we performed a high-throughput siRNA screen
84 targeting membrane trafficking proteins. Our results identified proteins involved in
85 vesicle trafficking between early endosomes, the trans-Golgi network (TGN) and the
86 plasma membrane that reduce transfer of HIV-1 from DC to T-cells. We show that
87 HIV-1 is dependent on a functioning endocytic pathway, disruption of which results
88 in an accumulation of virus in intracellular vesicles, blocking trafficking of the virus to
89 the virological synapse.

90

91 **RESULTS**

92 **siRNA membrane trafficking library identified genes involved in HIV-1 *trans*-**
93 **infection between DC and T-cells.**

94 To identify the cellular trafficking pathways involved in the transfer of HIV-1
95 in *trans*-infection from DC to CD4+ T-cells, a siRNA library targeting 140 membrane
96 trafficking genes was utilised. SMARTpool siRNA were transfected into MDDC 48
97 hours before infection with full-length CXCR4-tropic HIV-1 (R9) and co-cultured with
98 SUPT1 cells at 1:1 ratio. HIV-1 infected SUP-T1 cells were analysed by flow cytometry
99 48 hours later. No infection was detected in SUP-T1 cells inoculated with a HIV-1
100 fusion-mutant control (Figure 1a). Non-target siRNA were used to compare infection
101 levels and showed < 20% variation between replicates (Z score = 1.5 s.d.), therefore
102 the lower assay cut-off point was set at 20%.

103 In the primary screen, the knockdown of 16 genes induced a reduction in
104 HIV-1 *trans*-infection greater than or equal to 20%, whereas 25 genes showed an
105 increase in viral *trans*-infection by over 50% (Figure 1b). The primary Hits were
106 reproduced in two donors using autologous CD4+ T-cells activated with IL-2 and PHA.
107 Nine Hits showed a reproducible reduction in HIV-1 transfer: *AP1M1*, *AMPH1*, *ARF1*,
108 *BIN1*, *EPN3*, *PAK1*, *RAB7L1*, *RAB8A* and *WASF1* (Figure 1c). Hits that resulted in an
109 increase in HIV-1 transfer included *AP2M1*, *CLTB*, *CLTC*, *EPS15*, *GRB2*, *HIP1*, *RAB1A*,
110 *RAB2*, *ROCK1*, *VAV2*, *EFS*, *MAPK8IP2*, *DNM3* (Figure 1d).

111 **Efficient HIV-1 *trans*-infection requires vesicle trafficking at the plasma membrane**

112 To understand potential relationships between the genes selected in the
113 siRNA screen, gene-annotation enrichment analysis was used to identify common

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114 interactions between the candidate genes that may be involved in the *trans*-
115 infection of HIV-1 between DC and T-cells. Analysis of the siRNA candidates was
116 carried out for cellular compartments and biological processes (Table 1). Our results
117 show that the genes required for optimal viral transfer are primarily involved in
118 endocytic compartment regulation, whereas genes that restrict viral transfer are
119 largely involved in clathrin-coat mediated endocytosis and actin-dependent
120 processes at the plasma membrane. Taken together the data suggests that
121 preventing viral uptake via clathrin coated vesicles enhances viral transfer, which is
122 likely due to increased retention of virus on the cell surface. This finding is in
123 agreement with studies that show HIV-1 transmitted in *trans* between DC and T-cells
124 from the surface of DC (12, 13). In contrast, genes required for efficient *trans*-
125 infection are strongly associated with cytoplasmic membrane bound vesicles and
126 vesicle-mediated transport supporting the view that HIV-1 is sequestered into
127 intracellular virus-containing compartments (VCC) (15, 16, 33).

128

129 ***ARF1, BIN1, RAB7L1 and RAB8A are required for HIV-1 trans-infection***

130 The siRNA library used to identify target genes is comprised of a set of four
131 separate siRNA sequences which target different regions of the same gene; these are
132 pooled to reduce the potential off-target effects of siRNA transfection. The
133 knockdown of the pooled siRNA typically reflects the most functional siRNA within
134 the pool. Therefore, the four individual siRNA can be analysed for their ability to
135 reduce viral transfer to validate whether the observed phenotype is a genuine on-
136 target effect.

137 The main aim of our study was to identify cellular pathways involved in the
138 delivery of HIV-1 to the VS to aid *trans*-infection; therefore, we focused our
139 investigation on the genes that facilitate the transfer of HIV-1 between DC and T-
140 cells. Each of the four siRNA were transfected individually into MDDC, infected with
141 HIV-1 (R9) and co-cultured with autologous T-cells for 48 hours. Of the final nine
142 candidates, three showed a reduction in transfer ($\geq 20\%$) in at least two of the four
143 individual siRNA, across four independent donors: *BIN1* (siRNA B and C), *RAB7L1*
144 (siRNA A, C and D) and *RAB8A* (siRNA A, and B) (Figure 2a). An average reduction of
145 50% in transfer was evident for ARF1 siRNA A, whereas ARF1 siRNA B produced a 17-
146 20% knockdown in viral transmission in 3 out of the 4 donors analysed. Thus, in
147 conjunction with the targeted reduction of ARF1 at the protein level, this result
148 indicates that ARF1 siRNA A was the most functional siRNA in the pool and it was
149 therefore decided to pursue this candidate further.

150 To determine the level of protein depletion in MDDC, cell lysates transfected
151 with 200 nM pooled siRNA (Figure 2b) targeting the entire length of the gene were
152 analysed by western blot. Knockdown was quantified by densitometry relative to
153 protein expression levels in non-target siRNA transfected lysates. An efficient
154 knockdown was achieved using pooled siRNA, 35% (± 17) reduction of protein
155 expression was observed for ARF1, 52% (± 6.6) for BIN1, 53% (± 23.4) for RAB7L1 and
156 54% (± 8.1) for RAB8A compared to non-target siRNA (Figure 2c).

157 To confirm whether siRNA is capable of reducing viral *trans*-infection
158 independent of viral strain, MDDC were transfected with the selected target siRNA
159 and infected with either R8BAL (CCR5-tropic) and R9 (CXCR4-tropic) HIV-1. A
160 significant reduction in viral transfer - ranging between 26-40% in R9 infected cells

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161 and 35-45% in R8BAL infected cells - was observed for all candidates demonstrating
162 that host factors involved in trafficking to the VS are shared for both CXCR4- and
163 CCR5-tropic strains of HIV-1 (Figure 2d and e). All MDDC transfected with pooled
164 siRNA remained > 80% viable compared to control cells, ensuring that the reduction
165 in transfer is not due to cellular toxicity of the siRNA transfection (Figure 2f). Further,
166 siRNA transfection of MDDC resulted in a marginal (< 5%) increase in DC maturation
167 marker CD83. Viral binding of p24 Gag also saw a marginal increase compared to
168 untreated and mock transfected cells, however HIV-1 internalisation was not
169 affected confirming the observed reduction in *trans*-infection is not due to
170 decreased binding or internalisation of the virus (data not shown).

171 Future experiments were conducted on selected candidate siRNA showing
172 evidence of protein knockdown and a reduction in viral *trans*-infection in at least two
173 of the four individual siRNA tested. siRNA candidate genes that failed to meet these
174 criteria (WASF1, EPN3, PAK1, and AMPH1) showed no evidence of a reduction in viral
175 *trans*-infection when MDDC were transfected with individual siRNA nor were we
176 able to detect a specific knockdown in protein expression, suggesting the previously
177 observed reduction in viral *trans*-infection maybe due to off-target effects of those
178 specific siRNA. Therefore, these genes were eliminated from further analysis along
179 with AP1M1 which showed high variability in the reduction of viral transfer between
180 donors. The final candidates included ARF1, associated with retrograde transport at
181 the Golgi and protein transport to endosomes (34, 35), BIN-1, known to form a
182 complex with dynamin to control vesicle transport and scission (36), RAB7L1 a
183 GTPase required for retromer recycling between the TGN and endosomes (37) and

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184 GTPase RAB8A involved in polarised vesicular trafficking to the plasma membrane
185 from TGN (38).

186

187 **Depletion of target proteins reduces virological synapse formation between MDDC**
188 **and CD4+ T-cells.**

189 The efficient *trans*-infection of HIV-1 from DC to T-cells is dependent on the
190 formation of VS, an adhesive structure that promotes viral transmission (39, 40). To
191 assess if the observed reduction in *trans*-infection was due to a reduction in VS
192 formation, siRNA transfected MDDC were infected with HIV-1 R9 or R8BAL and co-
193 cultured with autologous CD4+ T-cells. Imaging of the transfected MDDC revealed
194 that in the case of *BIN1* and *RAB7L1* siRNA transfected cells, HIV-1 R9 appeared to
195 accumulate in large cellular vesicles at the plasma membrane and did not form VS
196 with the T-cells in spite of apparent interactions between the two cell types. In
197 addition, *ARF1* and *RAB8A* depleted cells also appear to inhibit VS formation;
198 however, accumulation of virus can be seen in smaller vesicles at the cell periphery
199 (Figure 3a). Quantification of VS was similar in non-target siRNA, untreated and mock
200 transfected cells. All candidate siRNA had a 40-60% reduction in VS formation
201 between DC and T-cells when compared to non-target siRNA transfected cells (Figure
202 3b). Similar results were seen for R8BAL infected MDDC, a reduction in VS number
203 with T-cells was observed, however *BIN1* and *RAB7L1* transfected cells did not
204 accumulate virus in intracellular vesicles to the extent seen in R9 infected MDDC
205 (Figure 3c, 3d). In addition, we observed that LFA-1, a stabilising component of the
206 VS, did not become enriched at the interface between the MDDC and T-cells in the
207 absence of virus (data not shown). These data suggest that virus is targeted to

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cytoplasmic vesicles after entry into MDDC, however onward trafficking of virus to the plasma membrane is inhibited by depletion of the target genes, preventing VS formation and reducing efficient trans-infection between the DC and CD4+ T-cells.

211

The integrity of virus containing vesicles are compromised in BIN1 and RAB7L1 depleted MDDC cells.

CD81, a type II transmembrane protein, is one of the main tetraspanins recruited to the host cell membrane during HIV-1 *trans*-infection and is known to co-localize with HIV-1 containing compartments in macrophages and DC (4, 18, 41). To determine if target siRNA altered endogenous CD81 localisation in MDDC, transfected cells were labelled for CD81 (Figure 4a). In control cells (non-target siRNA) CD81 is found at the cell periphery with a faint perinuclear staining. In contrast, *ARF1* siRNA saw a reduction in CD81 positive vesicles that are evident within both the cytoplasm and at the cell periphery. *BIN-1* and *RAB7L1* depletion reduced CD81 vesicle number and size, whereas no significant difference was observed in cells depleted of RAB8A (Figure 4b-c). In all three cases, an accumulation of CD81 vesicles was observed within the cytoplasm not at the cell periphery (Figure 4a).

CD81 plays an important role in regulating viral *trans*-infection at the VS and depletion of the tetraspanin can reduce viral *trans*-infection (42). In light of previous findings, we assessed CD81 localisation during HIV-1 infection. As expected, we observed p24 Gag co-localisation with CD81 at the cell periphery in CD81 tetraspanin-enriched micro domains (TEM) at 4 hours post-infection in control cells. In transfected MDDC, we observed that the number of CD81 p24 Gag TEM's are

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232 reduced in *ARF1* depleted cells. In contrast, cells transfected with siRNA targeting
233 *BIN1*, *RAB7L1* and *RAB8A* saw both virus and CD81 at the cell periphery, however the
234 staining of the TEM was diffuse and lacked the structure of the TEM (Figure 4d-e).
235 This was confirmed by co-localisation data indicating that CD81 association with p24
236 was reduced in siRNA transfected cells (Figure 4f). Taken together, these data
237 suggest that trafficking of CD81 and p24 Gag to the cell periphery to form the TEM is
238 compromised by knockdown of *ARF1*, *BIN-1*, *RAB7L1* and *RAB8A* potentially
239 preventing the efficient *trans*-infection of virus via the VS.

240

241 **Retention of virus in endocytic compartments reduces HIV-1 transfer**

242 We hypothesised that the presence of virus and CD81 in cytoplasmic vesicles
243 and the disrupted trafficking of CD81 and p24 Gag to the plasma membrane by
244 target siRNA was due to retention in endocytic compartments. Therefore, we aimed
245 to trap virus in endosomal derived vesicles to establish if this directly affects viral
246 *trans*-infection to T-cells. MDDC were treated with endocytic inhibitors prior to
247 infection with R9 virus and the level of *trans*-infection was measured. We utilised
248 LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor known to block
249 macropinocytosis and the formation of early endosomes, and Bafilomycin A1, a
250 vacuolar type H⁺-ATPase (V-ATPase) that prevents endosomal acidification.

251 Inhibition with LY294002 resulted in a mild increase of HIV-1 *trans*-infection
252 (+ 20%) when compared to DMSO treated control cells. However, a 2-fold decrease
253 in *trans*-infection was observed in cells treated with Bafilomycin A1, indicating that
254 efficient viral transfer requires a functioning endocytic pathway (Figure 5a). This
255 reduction was not due to inhibitor toxicity, with MDDC remaining > 80% viable

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256 during treatment and subsequent infection (Figure 5b). To visualise any differences
257 between MDDC treated with LY294002 and Bafilomycin, infected MDDC were
258 analysed by confocal microscopy. HIV-1 was concentrated at the cell surface in cells
259 pre-treated with LY294002, which is in agreement with previous findings (13). In
260 contrast, virus accumulates inside intracellular vesicles in cells treated with
261 Bafilomycin A1 (Figure 5c) indicating that viral uptake into MDDC was not inhibited,
262 and retention within endocytic vesicles reduced *trans*-infection. Controls confirmed
263 that both horseradish peroxidase (HRP) taken into the MDDC via fluid-phase and the
264 lysosomal marker low-density lipoprotein (LDL) were lost in cells treated with the
265 PI3K inhibitor LY294002, as predicted. In contrast, LDL-Dil labelling was diffuse and
266 cytoplasmic in cells treated with Bafilomycin A1, suggesting a block in LDL-Dil uptake
267 by endosomes in the MDDC. On the contrary, HRP taken up via fluid-phase was less
268 affected, suggesting that unlike LDL, HRP is retained in endocytic-like compartments
269 (Figure 5d).

270 HIV-1 did not co-localise with organelle markers EEA1, Rab5, Rab7, Rab11,
271 LAMP2 or CHMP2B in either siRNA transfected or Bafilomycin A1 treated MDDC in
272 our experiments, suggesting that these HIV-1 positive compartments may be
273 intermediate vesicles devoid of characteristic markers.

274 Taken together, these data indicate that HIV-1 transfer is reliant on a
275 functioning endocytic pathway. Blocking virus in endosomal derived compartments
276 results in the accumulation of virus in cytoplasmic vesicles, which in turn reduces
277 viral transfer between MDDC and T-cells, as seen in siRNA transfected MDDC. In
278 addition, Bafilomycin A1 appears to block LDL-DIL but not HRP or HIV-1 uptake into

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279 MDDC, suggesting that HIV-1 is predominately trafficked to cellular compartments
280 that differ from those utilised by LDL.

281

282 **Downstream trafficking from early endosomes is compromised in MDDC**
283 **transfected with target siRNA**

284 Endosomal cargo has one of two fates; it is either recycled back to the cell
285 surface (i.e. transferrin) or directed to lysosomes for degradation (i.e. LDL). To
286 confirm that target siRNA are blocking endosomal trafficking in MDDC, cells were
287 transfected with pooled *ARF1*, *BIN-1*, *RAB7L1* and *RAB8A* siRNA and either stained
288 for early endosomes with early endosome antigen 1 (EEA1), incubated with Alexa
289 Fluor labelled Transferrin, or LDL-DIL, to assess the recycling and lysosomal
290 trafficking pathways respectively.

291 In non-target siRNA transfected MDDC, EEA1 is seen in numerous vesicles of
292 various sizes throughout the cell. MDDC transfected with siRNA against *ARF1*, *BIN1*
293 *and RAB8A* resulted in the formation of abnormal endosomes marked by a decrease
294 in both number and size. *RAB8A* siRNA resulted in more numerous, enlarged
295 vesicles evident at the cell periphery (Figure 6a-c).

296 In all instances labelled transferrin was found localised at the cell periphery,
297 with no discernible differences between control and siRNA transfected cells (Figure
298 6a, panel 2). However, a reduction in vesicle number and Alexa Fluor labelling was
299 observed in *BIN1*, *RAB7L1* and *RAB8A* transfected cells (Figure 6d-e).

300 In non-target siRNA or mock transfected control cells, LDL-DIL predominately
301 accumulates in lysosomes in the perinuclear region. In cells transfected with siRNA
302 targeting *ARF1*, LDL has accumulated in various sized vesicles in the cytoplasm

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303 (Figure 6a, panel 3). Knockdown of *BIN-1*, *RAB7L1* resulted in a reduction of LDL
304 containing vesicles within the cells indicating that the delivery of LDL-DIL to
305 lysosomes is significantly reduced (Figure 6a, f-g). *RAB8A*-silenced cells were also
306 found to have a reduced number of LDL vesicles; however, a diffuse staining is
307 evident within the cytoplasm, suggesting that LDL is taken into the cell but not
308 trafficked within the endolysosomal pathway.

309 These observations suggest *ARF1* regulates endosomal morphology and
310 vesicle formation and slows LDL trafficking to the perinuclear region but is
311 dispensable for the recycling of transferrin to the plasma membrane. *BIN1* and
312 *RAB7L1* also affect endosomal vesicle formation, resulting in the retention of vesicles
313 at the cell periphery and reducing downstream trafficking from endosomes,
314 evidenced by a reduction in both transferrin and LDL containing vesicles suggesting
315 *BIN1* and *RAB7L1* play a role in early endosomal protein trafficking. In contrast,
316 *RAB8A* depletion appears to increase early endosome size, although trafficking of
317 both LDL and transferrin is also reduced suggesting *RAB8A* actions is targeted more
318 downstream regulating protein trafficking after cargo has left the endocytic
319 compartment. The disruption of endosomal vesicle trafficking at or after the early
320 endosomal compartment by target siRNA creates a knock-on effect, altering
321 endocytic trafficking to lysosomes and recycling of cargo to the plasma membrane.
322 Taken together, the disruption of TGN-endosomal-plasma membrane trafficking
323 suggests that HIV-1 trafficking from internalised compartments relies of endosomal
324 sorting pathways to traffic to and accumulate at the VS and potentially within VCC at
325 the cell surface.

326

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327 **HIV-1 *trans*-infection requires retromer complex recycling of cargo**

328 Based on the findings that trafficking between key endosomal compartments
329 is compromised in siRNA targeted cells which in turn reduces HIV-1 trafficking to the
330 VS - we wanted to confirm whether *trans*-infection of HIV-1 was reduced when
331 trafficking to the plasma membrane from the TGN or early endosomes is
332 compromised. Several of our selected genes play a key role in endosomal sorting to
333 the TGN and plasma membrane with *RAB7L1* specifically involved in retromer
334 activity. In addition, a proportion of transferrin and its receptor are recycled in a
335 retromer-dependant manner to the plasma membrane (43). The retromer has also
336 been found to play a key role in HIV-1 Env trafficking and viral assembly (44). Thus,
337 we decided to investigate the role of the retromer complex in *trans*-infection using
338 siRNA targeting key components of the retromer complex, VPS26A and VPS35. HIV-1
339 *trans*-infection was significantly reduced in MDCC transfected with each of the
340 retromer siRNA (Figure 7a-b) from 25-50%. A more marked reduction was evident in
341 cells infected with CXCR4-tropic strain of the virus. A protein knockdown of
342 approximately 60% was confirmed for both VPS26A and VPS35 (Figure 7c-e), and no
343 reduction in cell viability was evident from siRNA transfection of the VPS genes.
344 Therefore, we were able to confirm endosomal sorting between the TGN and to the
345 plasma membrane is required for HIV-1 *trans*-infection.

346

347 **DISCUSSION**

348 DC perform an essential role in the transmission of HIV-1 to target CD4+ T-
349 cells promoting the spread of infection. Although there have been numerous
350 investigations into the role of DCs in *trans*-infection, the cellular trafficking pathways

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351 exploited by HIV-1 remain unclear. The identification of host cell factors and
352 intracellular pathways exploited by HIV-1 to aid *trans*-infection of T-cells will
353 facilitate the development of novel therapies and may reduce initial transmission of
354 HIV-1.

355
356 In this study we identified a number of host factors involved in *trans*-
357 infection of HIV-1 from DC to T-cells. By conducting a siRNA screen targeting
358 membrane trafficking proteins we identified four genes involved in efficient *trans*-
359 infection from DC to T-cells. Although, one similar shRNA/siRNA screen has been
360 conducted investigating the role of membrane trafficking in HIV-1 *trans*-infection,
361 none to date has focused on the genes we identified in the current study. In a recent
362 shRNA screen Menager and Littman (13) also identified *ARF1*, *ARF6* and *ARPC1B* as
363 reducing viral transfer and *CLTC*, *CLTB* and *AP2M1* enhanced viral transfer, however
364 the ability to draw direct comparisons between the two studies is complicated by the
365 fact that Menager uses shRNA technology in a screen that targets a different gene
366 library, several of them not included in the siRNA screen we utilised. The study then
367 proceeds to concentrate on TSPAN7 and DNM2 and their role in *trans*-infection at
368 the cell surface, whereas we have focused on the trafficking of internalised virus. In a
369 study using an identical siRNA screen, Wen *et al.* identify a number of common
370 genes such as *RAB7L1*, *AP1M1*, *BIN-1*, *ARPC1B*, *DIAPH1*, *ARF6*, *WASF1*, *CLTC* and
371 *VAV2* required for HIV-1 and M-PMV virus release from HeLa cells (45). Overall, there
372 is a high consistency of hits between previous screens conducted in DCs and our own
373 membrane trafficking screen, verifying our findings.

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374 Our initial siRNA screen shows that knocking down genes associated with
375 clathrin-coated vesicle formation enhanced *trans*-infection; this suggests that
376 restricting viral uptake into MDDC and retention of virus on the cell surface
377 promotes HIV-1 *trans*-infection. It has been previously demonstrated that soluble
378 CD4 protein is able to inhibit infection therefore proposing that virus particles bound
379 to the surface of the MDDC were the main source of *trans*-infection (12). In support
380 of this model, Menager *et al.* demonstrated that DNM2 and TSPAN7, which
381 coordinate actin nucleation and stabilization, had roles in restricting endocytosis and
382 maintaining virus on cellular dendrites enabling transfer (13, 46). On the other hand,
383 there is compelling evidence for the model that HIV-1 is sequestered in plasma
384 membrane-derived invaginated compartments induced upon HIV-1 uptake (33).
385 From this compartment, viral particles can be released to the VS to initiate *trans*-
386 infection (15-17). We initially identified 9 genes from the siRNA screen that reduced
387 *trans*-infection. These genes were predominately associated with cytoplasmic,
388 membrane-bound vesicles with direct involvement in vesicle-mediated transport and
389 membrane organisation, thus supporting a requirement for membrane-bound
390 vesicles in HIV-1 *trans*-infection. These results provide evidence for both viral
391 transmission via the cell surface and trafficking via intra-cellular compartments to
392 promote *trans*-infection in MDDC.

393 Although the use of primary MDDC and CD4⁺ T-cells is a representative
394 model of HIV-1 *trans*-infection, employing methods such as siRNA transfection
395 within established MDDC has its limitations. Generally, 50% transfection efficiency is
396 achieved, which in turn does not completely block reduction of *trans*-infection within
397 these cells. However, partial knockdown is still capable of producing a strong

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398 phenotype and the study of these pathways in primary cells is essential to
399 uncovering underlying mechanisms of *trans*-infection and is critical for investigating
400 and identifying such cellular processes.

401 In this study, we concentrate on studying genes required for efficient viral
402 *trans*-infection and therefore aim to investigate how internalised virus is trafficked
403 to the VS. We demonstrate that the reduction in viral *trans*-infection observed from
404 depletion of four genes: *ARF1*, *BIN1*, *RAB7L1* and *RAB8A* is due to the apparent
405 retention of virus in intra-cellular vesicles and a reduction in virus accumulation at
406 the VS between DC and T cells. MDDC are able to capture and store HIV-1 virions in
407 invaginations at the plasma membrane (9, 15). Live-imaging shows viral puncta are
408 trafficked into enclosed intracellular compartments (47), whether these
409 compartments are enclosed or remain accessible to the cell surface is still at matter
410 of debate (15, 16). The integrity and formation of intracellular compartments are
411 believed to be regulated by membrane trafficking processes (17). Based on this data
412 we propose that the reduction in VS formation observed in siRNA-treated MDDC
413 disrupts the regulation and trafficking of intra-cellular compartments resulting in the
414 retention of viral particles within intra-cellular vesicles, preventing onward
415 trafficking to the VS and therefore inhibiting viral *trans*-infection.

416 VS formation and HIV-1 spread relies on the interaction of MDDC and
417 recipient T-cells, triggering the active polarisation of organelles and cell surface
418 proteins. One such component, LFA-1, has been shown to induce T-cell polarisation
419 towards the VS to induce efficient viral T-cell-to-T-cell spread (48). In the context of
420 VS formation between DC and T-cells it has been reported that cell-to-cell contacts
421 are not increased by the presence of HIV-1 and the formation of the VS was

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422 decreased by 60% when the interaction between ICAM-1 and LFA-1 was blocked
423 (49). Our findings agree with this data, we also observe several T-cells interacting
424 with HIV-1-infected siRNA-transfected MDDC; however accumulation of LFA-1 at the
425 VS was only evident in the presence of HIV-1 p24 Gag. These data suggest that by
426 blocking trafficking of HIV-1 to the cell periphery, enrichment of LFA-1 at the MDDC-
427 T-cell interface is also prevented, restricting VS formation. It may be the case that
428 virus alone is not the only trigger for VS formation and it is plausible that by blocking
429 trafficking of HIV-1 to the cell surface in MDDC we may also be preventing the
430 recruitment of other key components to form efficient VS.

431 We also observe the retention of endogenous CD81 in cytoplasmic vesicles
432 and a reduction of localisation at the cell periphery. In addition, at 4 hours post-
433 infection, TEMs are reduced or disrupted and potentially affecting the recruitment
434 and budding of HIV-1 at the VS. The tetraspanin CD81 co-localises with HIV-1 within
435 VCC (4, 18, 41) and accumulates at the VS promoting viral *trans*-infection, preventing
436 cell-to-cell fusion and providing a platform for viral budding (50, 51). Our results are
437 consistent with these findings suggesting that trafficking of CD81 within MDDC to
438 the plasma membrane and recruitment to TEMs, along with HIV-1, are required for
439 *trans*-infection. This is supported further by a study showing that blocking CD81 with
440 specific antibodies reduces VS formation (52). Although conversely, Kremontsov and
441 colleagues show that direct depletion of CD81 actually enhances viral transmission
442 between HeLa and Jurkat cells (53). The different outcomes observed in these
443 studies may reflect the different methods and cell types employed to target CD81
444 and reduce its presence at the VS. Our data supports the former approach where
445 CD81 is still present within the cell, but is prevented from forming functioning TEMs

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446 at the cell periphery, whereas actual depletion of CD81 from cells may have a
447 number of downstream effects, altering normal cell function.

448 Overall, we demonstrate that targeting host factors that regulate endocytic
449 compartments and vesicle trafficking to the plasma membrane within MDDC results
450 in the disruption of trafficking of CD81 and virus to the VS reducing *trans*-infection.

451 Our results show that upon disruption of target genes, protein trafficking to
452 lysosomes and recycling of transferrin to the plasma membrane is reduced: this
453 suggests that endosomal sorting and recycling to the plasma membrane are closely
454 linked to *trans*-infection in MDDC.

455 In conjunction with other ARF proteins, ARF1 plays an important role in the
456 regulation of recycling endosome morphology and recycling pathways, however
457 depletion of the gene was not found to directly affect the recycling of transferrin
458 receptor (54, 55). Depletion of ARF1 in our study is consistent with this role in
459 protein recycling in MDDC, altering endosomal morphology but not affecting
460 recycling of transferrin to the plasma membrane. In the context of infection, HIV-1
461 ability to mediate the down-regulation of MHC-1 is achieved by targeting AP-1 and
462 ARF1 activity (56) resulting in the accumulation of MHC-1 in the TGN or endosomes
463 (57). HIV-1 Vpu also targets the same pathway (58, 59) to counteract tetherin,
464 known to block the release of progeny virus from the cell (60). This data in
465 conjunction with the fact that ARF1 binding partner AP1M1 was originally identified
466 as a potential gene required for *trans*-infection in our screen, supports the idea that
467 the same recycling pathway could be utilised for the successful *trans*-infection of
468 HIV-1 in DC. Depletion of ARF1 is likely to impact on the morphology of virus
469 containing compartments and recycling of internalised virus to the cell surface,

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470 which in turn reduces the accumulation of virus at the VS and therefore *trans*-
471 infection.

472 BIN-1 is a key player in membrane remodelling during endocytosis and
473 endosomal sorting, essential for the formation of plasma invaginations in muscle
474 tissue (61). BIN-1 mutants were found to both impair membrane tabulation and
475 cause compact membrane curvature (62). Our findings support these data: depletion
476 of BIN-1 in MDDC reduces endosomal size, producing small round vesicles
477 preventing downstream trafficking. A role for BIN-1 in HIV-1 infection is supported
478 further by a study that identifies the up-regulation of BIN-1 in CD4+ and CD8+ T-cells
479 from *ex vivo* patients (63). Based on this, we propose that BIN-1 is required for the
480 efficient formation and function of plasma membrane invaginations and endosomal
481 sorting that assist the trafficking of HIV-1 to the VS.

482 RAB7L1 is also found to have a role in intracellular trafficking and the
483 endosomal sorting of lysosomal bound membrane proteins (64). Again, our results
484 support a similar role for RAB7L1 in MDDC, the transport of both LDL and transferrin
485 was impaired in RAB7L1 depleted MDDC, suggesting that trafficking from endosomal
486 compartments is compromised. The finding that RAB7L1 along with AP1M1 are
487 involved in HIV-1 Gag trafficking and virion budding in the activated macrophage cell
488 line MM6 and CD4+ Jurkat cells (65), supports a role for RAB7L1 in the recruitment
489 of HIV-1 particles in MDDC to the VS to assist viral budding at the cell surface.

490 RAB8A is known to control vesicular transport and promote membrane
491 protrusions, which can be inhibited by blocking membrane recycling (66) agreeing
492 with our findings. Knockdown of RAB8A by siRNA in previous studies was found to
493 inhibit HIV-1 replication in Hela P4/R5 cells and directly interact with nef, env and

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494 gag-pol (67). Therefore, it seems plausible that the depletion of RAB8A in MDDC
495 inhibits membrane recycling and therefore membrane protrusions, reducing HIV-1
496 *trans*-infection. The data also supports the idea that HIV-1 taken up by MDDC could
497 rely on the same recycling pathways to traffic to the cell membrane to accumulate
498 virus at the VS.

499 The data presented in this study clearly points to a role for endocytic
500 recycling pathways in HIV-1 *trans*-infection; therefore we investigated the retromer
501 complex implication in the trafficking of HIV-1 to the VS. Retromer-dependent
502 protein sorting pathways provide an opportune target for a variety of viral and
503 bacterial pathogens (68, 69). For instance, HIV-1 envelope protein and herpesvirus
504 saimiri, a T-lymphotrophic tumour virus, bind the retromer to aid infection and viral
505 release (44, 70), whereas influenza A M2 protein escapes degradation via
506 transportation from early endosomes to the TGN (71). Our data confirms a role for
507 the retromer in DC-mediated HIV-1 *trans*-infection and exploitation of recycling
508 pathways by the virus to achieve efficient transfer between cells.

509 We hypothesise that VCC and VS formation is dependent on the retromer-
510 dependent endocytic-TGN-plasma recycling pathway. By exploiting the retromer
511 pathway, internalised viral particles can be subverted to the plasma membrane
512 where virus becomes sequestered to promote VS formation and enable *trans*-
513 infection between MDDC and T-cells (Figure 8).

514 DC are among the most important cellular targets in early HIV-1 transmission.
515 HIV-1 is thought to accumulate in 'viral endosomes' where the virus is able to exploit
516 a pathway essential for the delivery of components to the immunological synapse
517 and activation of T-cells (4). Uptake into DC using this method not only allows

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518 efficient *trans*-infection to target CD4⁺ T-cells but also evades detection by the
519 immune system (27), the importance of which was shown *in vivo* using a humanised
520 mouse model (10, 11).

521 By using high-throughput siRNA screening we were able to identify *ARF1*,
522 *BIN1*, *RAB7L1* and *RAB8A* that are essential for endosomal trafficking between the
523 TGN and early endosomes and co-ordinated transport to the plasma membrane in a
524 retromer-dependent manner. Thus, we identify key cellular trafficking proteins
525 exploited by HIV-1 in DC to efficiently disseminate virus to target T-cells promoting
526 *trans*-infection. A better understanding of the role of these proteins in viral transfer
527 to T-cells may serve as potential candidates for targeted therapy to control the
528 transfer of HIV-1 between DC and T-cells *in vivo*.

529

530 **EXPERIMENTAL PROCEDURES**

531 **Ethics statement**

532 Peripheral blood mononuclear cells (PMBC) were derived from buffy coats obtained
533 from healthy blood donors, anonymously provided by the Welsh Blood Service, UK.
534 Written informed consent for the use of buffy coats for research purposes was
535 obtained from blood donors and the use of patient samples and procedures were
536 approved by the local research ethics committee at Cardiff University.

537

538 **Cells**

539 Primary cells were isolated from PMBC of healthy blood donors using magnetic bead
540 selection (Miltenyi Biotech). CD14⁺ monocytes were differentiated into immature

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541 monocyte-derived dendritic cells (MDDC) with IL-4 and GM-CSF, as described
542 previously (72, 73).

543 CD4⁺ T-cells were isolated using CD4⁺ magnetic beads (Miltenyi Biotech) and
544 maintained in the presence of IL-2 and activated 4 days before use with 2 µg/mL
545 phytohemagglutinin (PHA). SUP-T1 T-lymphoblasts and 293T human embryonic
546 kidney (HEK) cells (obtained from NIH AIDS Research & Reference Reagent Program)
547 were maintained in supplemented RPMI 1640 or DMEM respectively.

548

549 **Viral stock production**

550 Viral stocks were produced by transfection of HEK293T cells with calcium phosphate
551 DNA precipitation of proviral plasmids encoding full length HIV-1 X4 and R5 provirus,
552 pR9 and pR8BAL respectively (plasmids provided by Trono D, EPFL, Lausanne).
553 Infectious titres were determined by titration onto SUP-T1 cells and quantification of
554 HIV-1 p24 Gag by ELISA using the Lenti-X p24 rapid titre kit (Clontech).

555

556 **Antibodies and reagents**

557 HIV-1 p24 was detected using anti-HIV-1 core antigen antibody-FITC (KC57-FITC –
558 Beckman Coulter), and actin labelled with Cytopainter Phalloidin-iFluor-555 (abcam).
559 Protein knockdown was detected by immunoblotting using rabbit anti-ARF1, anti-
560 BIN1, anti-RAB8A, mouse anti-Rab7L1 (abcam), and Actin (Merck) followed by
561 secondary HRP conjugated goat anti-rabbit and anti-mouse (DAKO). Confocal
562 microscopy was carried out using primary antibodies, anti-human CD81-APC (BD),
563 anti-EEA1, anti-CHMP2B, anti-LAMP1, anti-Rab7, anti-Rab11, anti-Rab5 (abcam).
564 Horse radish peroxidase (HRP) uptake was detected using anti-HRP (Jackson

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Immunolaboratory). All unlabelled primary antibodies were detected with secondary anti-rabbit Alexa Fluor 546 (Life technologies). Pharmacological inhibitors: LY294002, Bafilomycin A1, Indinivir (Sigma Aldrich) were used at 50 μ M, 0.5 μ M and 2 μ g/mL respectively.

RNAi screen in MDDC

siRNA screen was performed using a commercially available SMARTpool ON-TARGET library containing 140 membrane trafficking genes (Dharmacon-GE Healthcare, also see table S1). MDDC (1×10^5 cells/well) seeded in 96 well plates were reverse transfected twice, 24 hours apart, with 200 nM of pooled siRNA or with control siRNA (SMARTpool ON-TARGET Non-target siRNA, Dharmacon-GE Healthcare) using HiPerFect transfection reagent (Qiagen) in serum free media. After 48 hours, MDDC were infected with 20-30 ng p24 Gag HIV-1 R9 by spinoculation and co-cultured with SUP-T1 or CD4⁺ T-cells pre-stained with Celltrace™ Far Red (Invitrogen) in the presence of Indinivir 2 μ g/mL (Sigma) for a further 48 hours, as previously described (72).

Flow Cytometry

Phenotyping of primary cells was performed by washing MDDC and CD4⁺ T-cells in ice cold buffer (PBS, 0.5% BSA) before staining for HLR-DR, CD209, CD83 or CD14-APC (BD). SUP-T1 and autologous T-cells were labelled with CD4 and CD3-APC (BD). Cell viability was assessed using LIVE/DEAD stain 1:1000 (Life Technologies) in PBS,

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589 as per manufacturer's instructions. Infected MDDC and CD4+ T-cells were fixed in 2%
590 PFA and stained for HIV-1 p24 Gag-FITC after permeabilisation with 1x PhosFlow
591 buffer (BD). Stained samples were washed twice before measurements were taken
592 on the FACS Calibur (Beckton Dickinson) Canto II and analysed using Flowjo V10
593 software (Flowjo, LLC).

594

595

596 **Transfer Assay**

597 MDDC (1 x10⁵ cells/well) were reverse transfected twice, 24 hours apart, with 200
598 nM of pooled or individual siRNA using HiPerFect transfection reagent (Qiagen) in
599 serum free media. After 48 hours, MDDC were infected with 5-10 ng p24 Gag HIV-1
600 R9 or 2-5 ng p24 Gag HIV-1 R8BAL by spinoculation for 2 hours and co-cultured with
601 CD4+ T-cells pre-stained with Celltrace™ Far red (Invitrogen) at 37°C for a further 48
602 hours.

603

604 **Western Blot Analysis**

605 At 72 hours post-transfection cells were lysed with 1x cell lysis buffer (Cell Signalling)
606 and supernatants harvested and reduced. Cell lysates were separated on a 4-12%
607 SDS-PAGE gel and run next to a PAGEruler (Thermofisher) before being subjected to
608 western blotting followed by ECL detection and densitometry analysis (Mylmage
609 Analysis, ThermoScientific).

610

611 **Uptake Assays**

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612 Transfected MDDC were incubated with HRP (Sigma) 10 mg/mL for 1 hour at 4°C
613 prior to fixation on coverslips using 2% Paraformaldehyde (PFA) and labelled using
614 indicated antibodies.

615 Transfected MDDC (1×10^5) were seeded onto poly-L-lysine coverslips and placed at
616 4°C for 10 minutes prior to the addition of either 12 µg/mL LDL-DIL (Life
617 Technologies) for 4 hours or 25 µg/mL Transferrin Alexa Fluor 488 (Life Technologies)
618 for 30 minutes, both at 37°C. Cells were fixed in 1% PFA and nuclei labelled with
619 TOPRO-3 (Life Technologies).

620

621 **Inhibition assays**

622 Inhibitors LY294002 (50 µM) and Bafilomycin A1 (0.5 µM) were added to MDDC 1
623 hour prior to and during infection with R9 HIV-1. DMSO was used as a control at
624 equal concentrations. Cells were either seeded on coverslips and fixed in 2% PFA for
625 confocal imaging, or washed and co-cultured with CD4⁺ T-cells for 48 hours at 37°C
626 for analysis via flow cytometry.

627

628 **Virological synapse assay**

629 MDDC transfected with siRNA were infected with HIV-1 for 2 hours prior to
630 incubation with CD4⁺ T-cells on Poly-L-Lysine coverslips at 1:1 ratio for 40 minutes at
631 37°C. Fixed cells (2% PFA) were labelled for Actin and p24 Gag-FITC and viewed on
632 the confocal microscope. Virological synapse formation was counted if an
633 accumulation (approx. 50% or greater) of p24 Gag was evident at or adjacent to the
634 junction between T-cells and MDDC. T-cells were identified by their smaller size and
635 less cytoplasmic content in comparison to larger MDDC.

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636

637 **Confocal Immunofluorescence**

638 Cells were adhered to Poly-L-Lysine coverslips (Corning), fixed in 2% PFA,
639 permeabilised with 0.05% saponin and stained with indicated primary antibodies in
640 PBS/0.2% BSA/0.05% saponin followed by Alexa Fluor labelled secondary antibodies
641 (1:400) when necessary. TOPRO3 in PBS (1:1000) was used to stain nuclei (Life
642 Technologies). Confocal microscopy analysis was carried out using Zeiss LSM710
643 using 100x oil objective with 488, 546, 633nm acquired sequentially using ZENlite
644 software (Zeiss). All confocal images represent a single plane. Co-localisation analysis
645 was performed using Zenlite software (Zen Blue) using the co-localisation function.

646

647 **Bioinformatics - Protein Interrelationship mapping**

648 RNAi screen candidates were enriched using DAVID to identify significant gene
649 ontology (GO) terms and a protein-protein interaction network was visualised using
650 EnrichmentMap (Bader Lab) plug-in for Cytoscape 3.3.3; the top 5 significant values
651 were reported. The minimum confidence score was set at 0.005 (74-77).

652

653 **Image Analysis**

654 Image analysis was performed using ImageJ software (NIH) and analysed with Excel
655 software (Microsoft). A macro was designed to apply a set scale to all images
656 followed by the colour threshold to eliminate any background staining and the
657 particle analysis function was applied to quantify vesicles. Pixels were converted to
658 μM using the set scale.

659

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Statistics

Data was analysed using a two-sample T-Test, comparing non-target to targeted siRNA samples. A one sample T-test was used to compare siRNA transfer assays across donors. P-values <0.05, <0.005, <0.0005 were considered significant marked *, **and *** respectively. Data was analysed using Prism (Graphpad) software.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.P. and R.B. Methodology, R.B., V.P., C.M.N. and S.C. Investigation and validation, R.B. Formal Analysis, R.B. and J.W. Writing –original draft and visualisation, R.B. Writing – review and editing, S.C., V.P. and C.M.N. Funding acquisition and supervision, V.P.

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FIGURE LEGENDS

Figure 1. The procedure and results of the siRNA screen used to investigate *trans*-infection of HIV-1 between MDDC and T-cells.

- A) Schematic of the method used to study the effects of siRNA knockdown on HIV-1 *trans*-infection between DC and CD4+ T cells.
- B) Results of siRNA screen on HIV-1 *trans*-infection between MDDC and CD4+ T-cells. Red dashed lines indicate the assay cut off of -20% and +50% for non-specific variation of the assay. siRNA that reduced or increased HIV-1 *trans*-infection above or below the cut-off point (HITS) are listed in the grey boxes.
- C) Identification of genes that reduced HIV-1 *trans*-infection between MDDC and T-cells. Results from initial screen conducted in SUPT1 cells (○) are shown in

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962 combination with repeats conducted in with autologous CD4+ T-cells (●). Mean
963 and SD of three independent donors shown. Only genes with a mean percentage
964 below that of the non-target siRNA are shown.

965 D) Identification of genes that increase HIV-1 *trans*-infection between MDDC and T-
966 cells. Results from initial screen conducted in SUPT1 (○) cells are shown in
967 combination with two repeats conducted in with autologous CD4+ T-cells (●).
968 The mean and SD of three independent donors was calculated per gene. Only
969 genes with a mean above that of the non-target siRNA are shown.

970

971 **Figure 2. ARF1, BIN1, Rab7L1 and RAB8A regulate DC T-cell HIV *trans*-infection**

972 A) Validation of siRNA knockdown on *trans*-infection against four individual siRNA
973 from each candidate gene. Percentage of HIV-1 transfer is normalised to non-
974 target siRNA set at a value of 1.0. Each point represents an individual donor.
975 The mean and SD \pm is shown. * $p < 0.05$, ** $p < 0.005$.

976 B) Western blot analysis of pooled siRNA knockdown in MDDC at 72 hours post-
977 transfection with *ARF1*, *BIN1*, *RAB7L1* and *RAB8A* siRNA performed in triplicate,
978 untreated MDDC and non-target siRNA. Actin is used as a loading control.

979 C) Densitometry quantification of protein expression levels for ARF1, BIN1, RAB7L1
980 and RAB8A. Protein expression levels for siRNA transfected MDDC normalised
981 to actin loading control. All values are relative to non-target siRNA transfected
982 lanes (Set at 1.0). Mean and SD \pm shown, $n=3$.

983 D) The effects of final target siRNA on HIV-1 *trans*-infection infected with CXCR4
984 (R9). The reduction in viral transfer is measured relative to Non-target siRNA.
985 Mean and SD shown for each sample ($n = 5$) * $p < 0.05$, ** $p < 0.005$.

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- 986 E) The effects of final target siRNA on HIV-1 *trans*-infection infected with CCR5
 987 (R8Ba). The reduction in viral transfer is measured relative to Non-target
 988 siRNA. Mean and SD shown for each sample (n = 5) * p < 0.05, ** p < 0.005.
- 989 F) The effects of ARF1, BIN1, RAB7L1, RAB8A siRNA transfection on the viability of
 990 MDDC at 48 hours post-transfection. All samples compared to untreated MDDC.
 991 Cell viability is shown as a percentage. Mean ± SD, n = 2.

992

993 **Figure 3. ARF1, BIN1, RAB7L1 and RAB8A are regulators virological synapse formation**
 994 **between HIV-1 infected MDDC and CD4+T-cells.**

- 995 A) Images of CXCR4 HIV-1 R9 (p24 green) infected, siRNA transfected MDDC
 996 interacting with CD4+ T-cells (identified with *). Actin = red, nuclei = blue.
 997 Scale= 10 µM.
- 998 B) Quantification of virological synapse formation between MDDC and CD4+ T-
 999 cells was counted in siRNA transfected MDDC infected with HIV-1 R9 and co-
 1000 cultured with autologous CD4+ T-cells. T-cells were identified as the smaller
 1001 cells with less cytoplasmic content compared to the larger MDDC in co-culture.
 1002 Data normalised to MDDC transfected with non-target siRNA. The mean and SD
 1003 of three independent donors (n = 500 cells) is shown. ** p < 0.05. ** p < 0.05.
- 1004 C) Images of CCR5 HIV-1 R8Ba (p24 green) infected, siRNA transfected MDDC
 1005 interacting with CD4+ T-cells (identified with *). Actin = red, nuclei = blue.
 1006 Scale= 10 µM.
- 1007 D) Quantification of virological synapse formation between MDDC and CD4+ T-
 1008 cells was counted in transfected MDDC infected with HIV-1 R8Ba and co-
 1009 cultured with autologous CD4+ T-cells. Data normalised to MDDC transfected
 1010 with non-target siRNA. The mean and SD of three independent donors (n = 300
 1011 cells) is shown. ** p < 0.05. ** p < 0.05.

1012

1013 **Figure 4. CD81 localisation and TEM formation is disrupted in MDDC transfected with**
 1014 **ARF1, BIN1, RAB7L1 and RAB8A siRNA.**

1015 A) The effects of target siRNA on CD81 staining and localisation in MDDC. CD81=
 1016 green, nuclei = blue. Scale = 10 μ M.

1017 B) Quantification of CD81 vesicles in target siRNA transfected MDDC compared to
 1018 non-target siRNA control (n = 110 cells, across three independent donors).
 1019 Mean and SEM shown* p < 0.05, *** p < 0.0005.

1020 C) Average size (μ M) of CD81 positive vesicles in MDDC transfected with target
 1021 siRNA compared to non-target siRNA (n = 150 cells, across three independent
 1022 donors). Mean and SEM shown. ** p < 0.005, *** p < 0.0005.

1023 D) Images of CD81 (red) and HIV-1 p24 Gag (green) in infected MDDC transfected
 1024 with non-target and target siRNA. Images show HIV-1 4 hours post-infection.
 1025 Nuclei = Red (spherical). Scale 10 μ M.

1026 E) Quantification of CD81 and p24 at tetraspanin enriched domains (TEM) in
 1027 infected MDDC at 4 hours post-infection. The mean percentage of cells with
 1028 HIV-1 p24 Gag localised at CD81 enriched TEMs is represented by black bars.
 1029 White bars represent the absence of CD81 enriched TEMs. Mean percentage
 1030 and SD is shown. N = 170 cells, across 2 independent donors.

1031 F) Co-localisation analysis of TEM in siRNA transfected MDDC compared to control
 1032 cells. The co-localisation coefficient of CD81 with HIV-1 p24 Gag is shown for
 1033 each condition. Mean and SEM \pm shown, n=11 fields analysed over 2
 1034 independent donors. **p < 0.005, *** p < 0.0005

1035

1036 **Figure 5. Retention of virus in endocytic derived compartments reduces HIV trans-infection**
 1037 **from DC to T-cells.**

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1038 **A)** The effect of LY294002 and Bafilomycin A1 treatment on HIV-1 transfer. MDDC
1039 were pre-treated with inhibitors overnight prior to infection with HIV-1 (R9)
1040 before co-culture with autologous CD4+ T-cells for 48 hours in triplicate in 2
1041 independent donors. Mean percentage (%) viral transfer and SD shown, *** $p <$
1042 0.0005.

1043 **B)** Percentage viability of MDDC after overnight incubation with LY294002 and
1044 Bafilomycin A1 at 0, 2 and 48 hours post-infection (pi). The percentage (%) of
1045 reduction in cell viability was assessed using a Live/Dead stain and analysed by
1046 flow cytometry. Mean and SD shown. Experiments performed in triplicate in 2
1047 independent donors

1048 The effect of inhibitor LY294002 and Bafilomycin A1 on HIV-1 localisation in
1049 MDDC. MDDC pre-treated with inhibitors were infected with HIV-1 for analysis
1050 by confocal microscopy. Labelling of p24 Gag HIV-1 = green, nuclei = blue. Scale =
1051 10 μ M.

1052 **C)** The effect of LY294002 and Bafilomycin A1 on LDL-DIL and HRP uptake into
1053 MDDC. Inhibitors added overnight before the addition of LDL-DIL = green, and
1054 HRP = red, nuclei blue. Scale = 10 μ M.

1056 **Figure 6. Endocytic trafficking is compromised in BIN1, RAB7L1 and RAB8A transfected**
1057 **MDDC.**

1058 The effect of target siRNA on vesicle trafficking in MDDC.

1059 **A)** MDDC transfected with ARF1, BIN-1, Rab7L1 and RAB8A siRNA for 48 hours
1060 were either labelled with either EEA1 for early endosomes (red, panel 1), or
1061 incubated with Transferrin (green, panel 2), for 20 minutes 37°C or LDL-DIL
1062 (green, panel 3) for 2 hours 37°C. Non-target siRNA was used a control. Nuclei
1063 in Blue. Scale = 10 μ M.

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- 1064 B) Quantification of EEA1 vesicles in MDDC transfected with target siRNA
 1065 compared to non-target siRNA control (n = 150 cells). Mean and SEM from
 1066 three independent donors shown, * p < 0.05, *** p < 0.0005.
- 1067 C) Average EEA1 vesicle size (μM) in MDDC transfected with target siRNA
 1068 compared to non-target siRNA control (n = 150 cells). Mean and SEM from
 1069 three independent donors shown, *** p < 0.0005.
- 1070 D) Quantification of the number of transferrin positive vesicles under each
 1071 condition compared to non-target control (n=150). Mean and SEM from three
 1072 independent donors shown. *p < 0.05.
- 1073 E) Measurement of the intensity of Transferrin in transfected MDDC under each
 1074 condition compared to non-target control (n=150). Mean and SEM from three
 1075 independent donors shown. *p < 0.05, **p < 0.005.
- 1076 F) Quantitative analysis of LDL-DIL containing vesicles (n = 120). Mean and SEM
 1077 from three independent donors shown, * p < 0.05, *** p < 0.0005.
- 1078 G) Intensity of LDL-DIL in transfected MDDC compared to non-target siRNA (n =
 1079 120). Mean and SEM from three independent donors shown, * p < 0.05.

1080

1081 **Figure 7. HIV-1 *trans*-infection requires retromer recycling to the plasma membrane.**

- 1082 A-B) The reduction in HIV-1 *trans*-infection between MDDC and CD4+ T-cells in
 1083 MDDC transfected with VPS26A and VPS35 via siRNA transfection. The
 1084 reduction in *trans*-infection is normalised to non-target siRNA for R9 (d) and R8-
 1085 BAL (e). Mean ± SD is shown, n = 4. *p < 0.05, ***p < 0.0001.
- 1086 C-D) Western blots showing the knockdown of VPS26A and VPS35 in MDDC,
 1087 performed in triplicate, compared to untreated cell lysate and non-target siRNA
 1088 transfected MDDC. Actin used as a loading control.

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1089 E) Quantification of protein knockdown of VPS26A and VPS35 in transfected
1090 MDDC relative to non-target lane. All lanes compared to corresponding Actin
1091 loading control (black bars). Mean and \pm SD shown, n = 3.

1092

1093 **Figure 8 Model for the roles of ARF1, BIN1, RAB7L1 and RAB8A in the endocytic pathway**
1094 **and vesicle formation in MDDC.**

1095 Molecules are internalised from the cell surface via endocytic vesicles that fuse with each
1096 other or existing endocytic vesicles to form early endosomes. The budding of vesicles
1097 containing cargo from early endosomes to the plasma membrane and trans-Golgi network
1098 (TGN) requires the activity of BIN1. TGN vesicles bud from the TGN surface and either fuse
1099 with each other or endocytic compartments. The TGN is responsible for sorting receptors
1100 from degradative compartments and delivers newly synthesised lysosomal enzymes in the
1101 form of lysosomal hydrolase via the mannose-6-phosphate receptor. Both transferrin and
1102 LDL are taken into the cell via clathrin-receptor mediated endocytosis. Transferrin and its
1103 receptor are recycled from early endosomes back to the plasma membrane. LDL is trafficked
1104 directly to lysosomes prior to release into the cytoplasm. The dynamic retrograde transport
1105 of vesicles between the TGN and endocytic compartment and the plasma membrane via the
1106 retromer and other trafficking pathways required depends on the activity of ARF1, RAB7L1
1107 and RAB8A. HIV-1 *trans*-infection between MDDC and CD4+ T-cells requires a homeostatic
1108 balance of the endocytic pathway. By blocking trafficking of molecules between early
1109 endosomes and the TGN and onward polarised transport of cargo to the plasma membrane,
1110 HIV-1 *trans*-infection is inhibited. Depletion of targeted proteins results in the accumulation
1111 of HIV-1 in intracellular vesicles that are unable to traffic to the virological synapse

1112

1113 **Table 1**

1114 **Network analysis statistical data**

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1115 A) Statistical Data (p- and q-values) for biological processes of genes inhibitory to HIV-1
1116 *trans*-infection. Values < 0.005 are displayed for each node name. The number of data sets
1117 included in the process are indicated under the dataset size heading.

1118 B) Statistical Data (p- and q-values) for biological processes of genes facilitating HIV-1 *trans*-
1119 infection. Values < 0.005 are displayed for each node name. The number of data sets
1120 included in the process are indicated under the dataset size heading

1121 C) Statistical Data (p- and q-values) for cellular compartments of genes inhibitory to HIV-1
1122 *trans*-infection. Values < 0.005 are displayed for each node name. The numbers of data sets
1123 associated with the cellular compartments are indicated under the dataset size heading.

1124 D) Statistical Data (p- and q-values) for cellular compartments of genes facilitating HIV-1
1125 *trans*-infection. Values < 0.005 are displayed for each node name. The numbers of data sets
1126 included in the process are indicated under the dataset size heading.

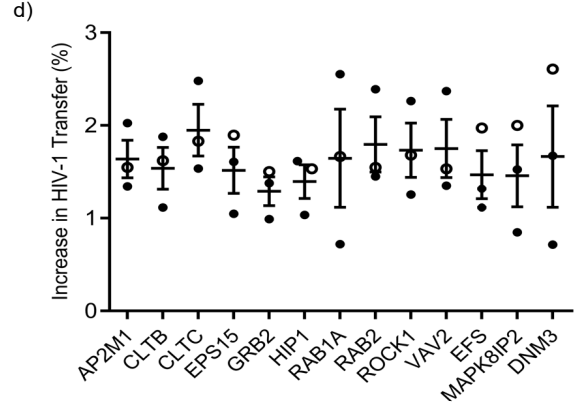
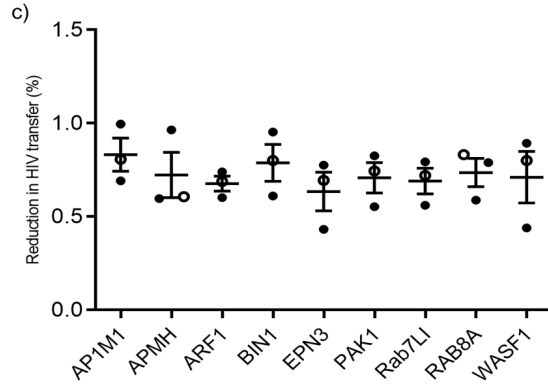
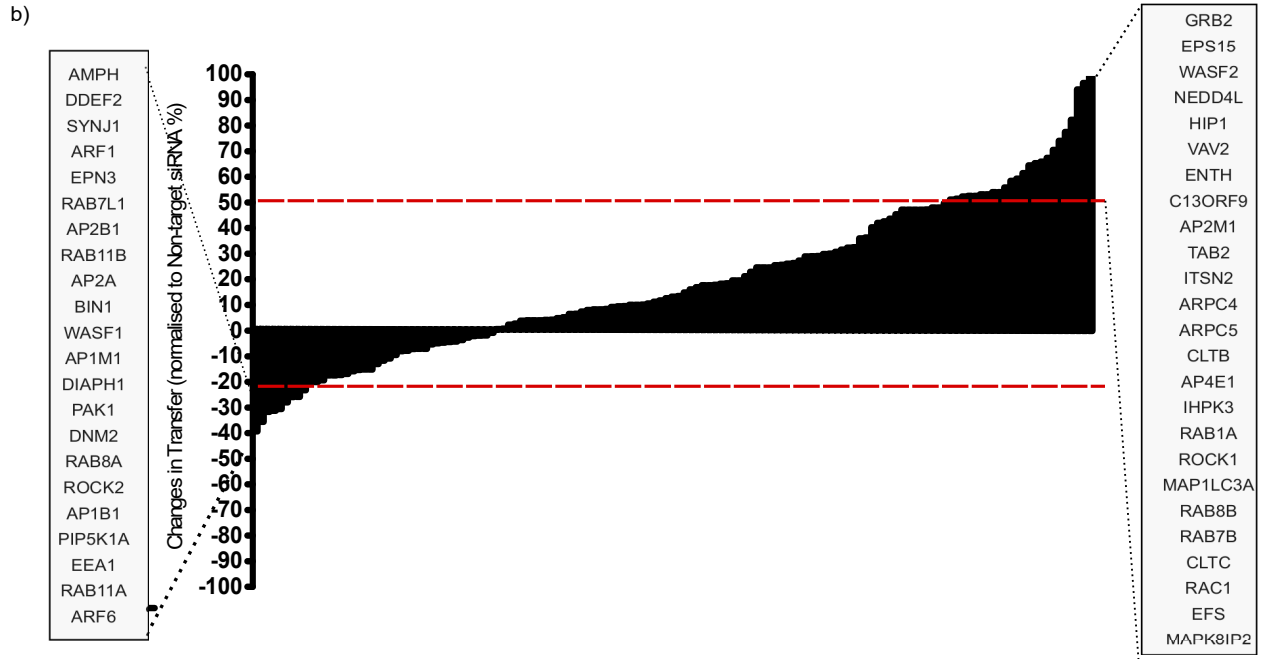
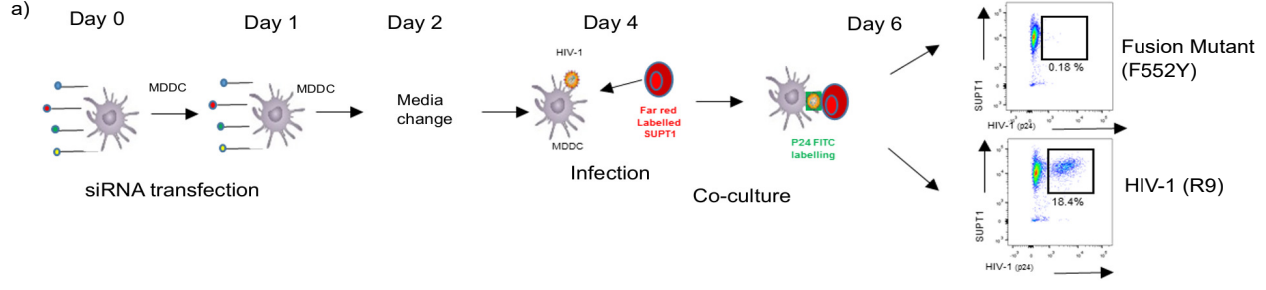
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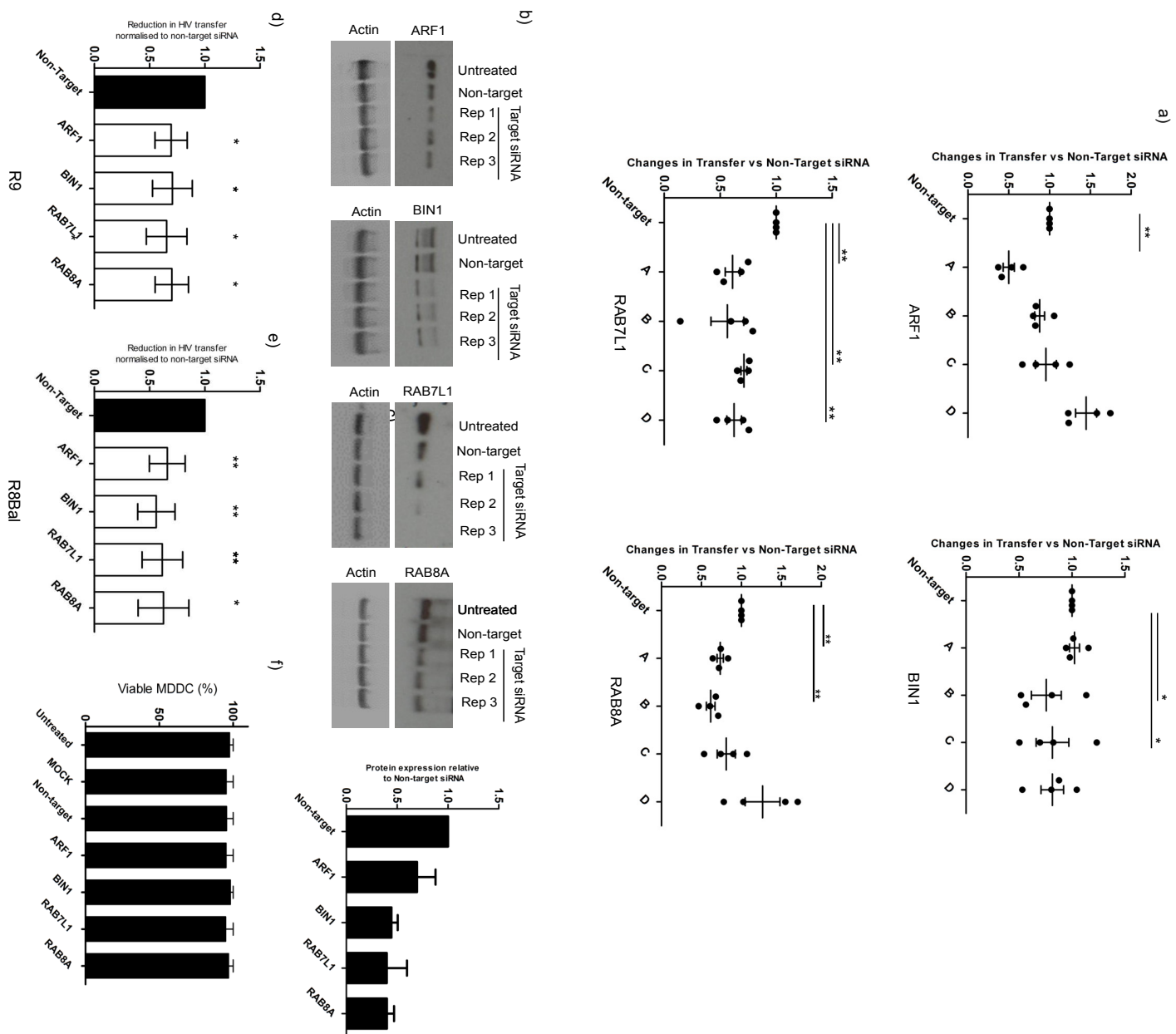
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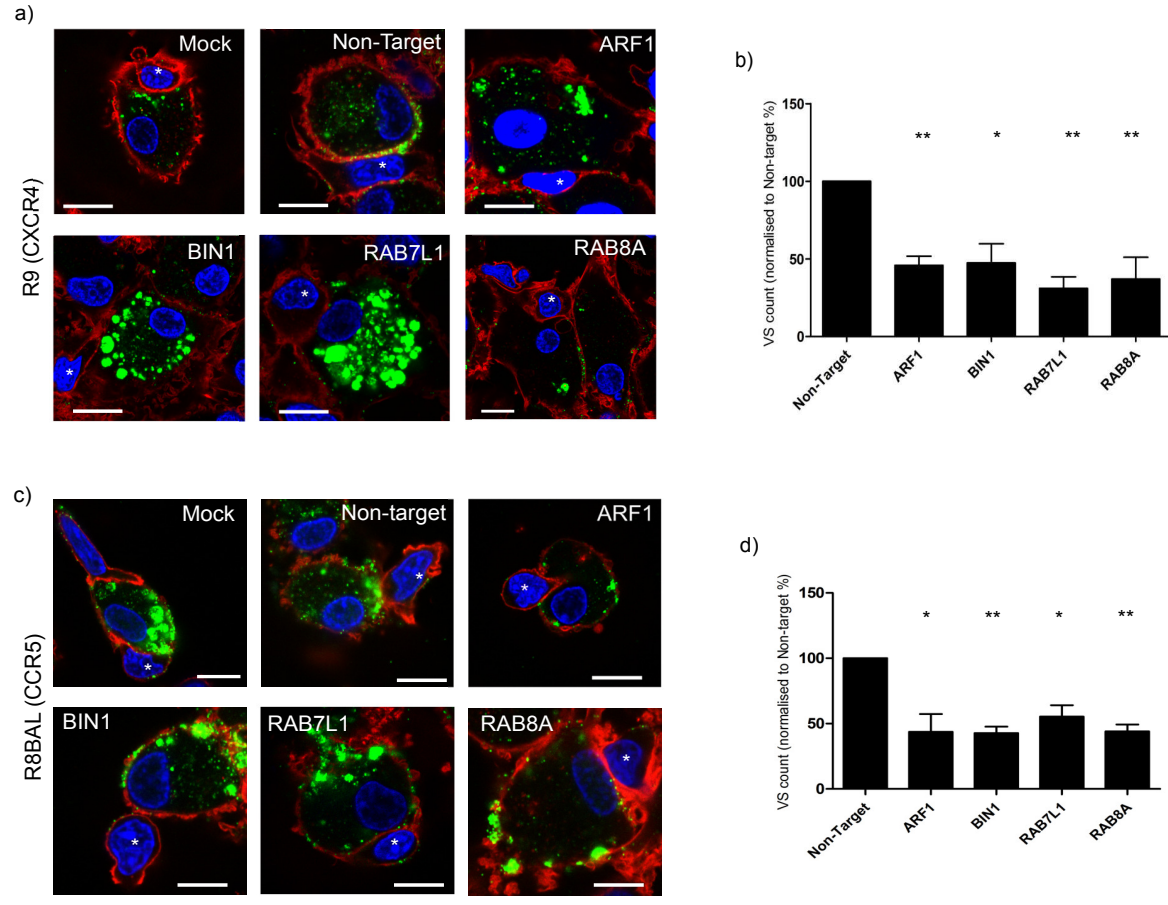
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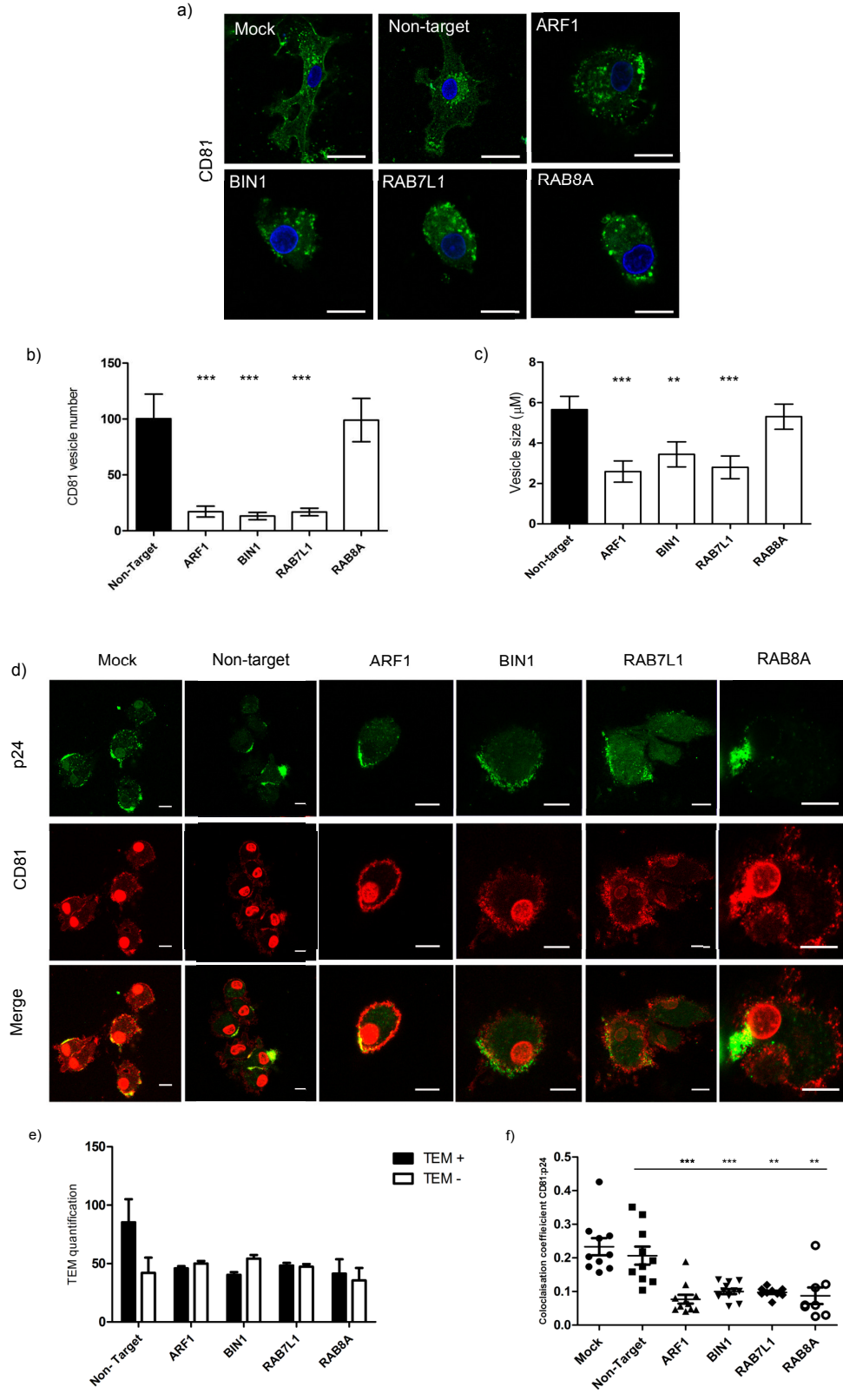
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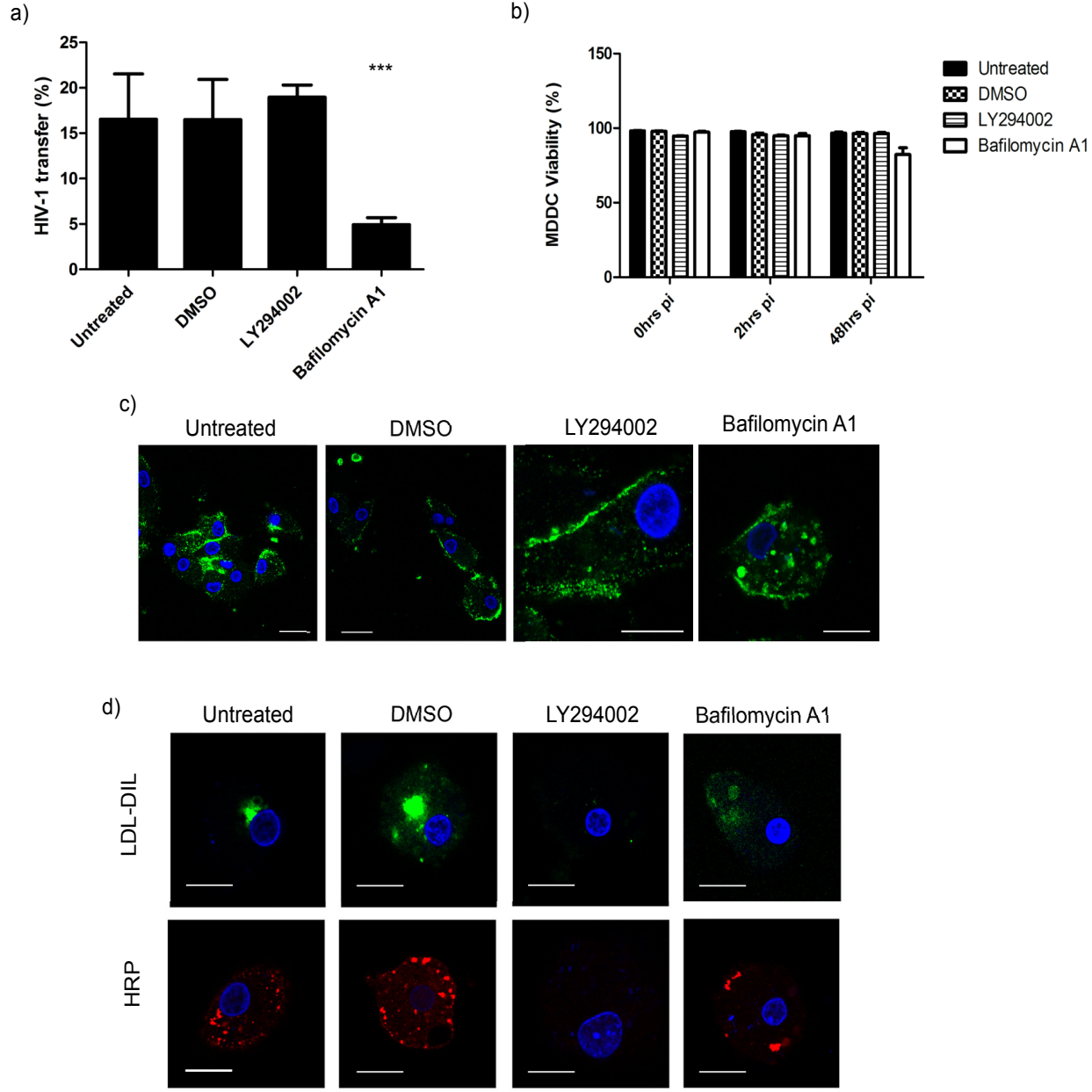
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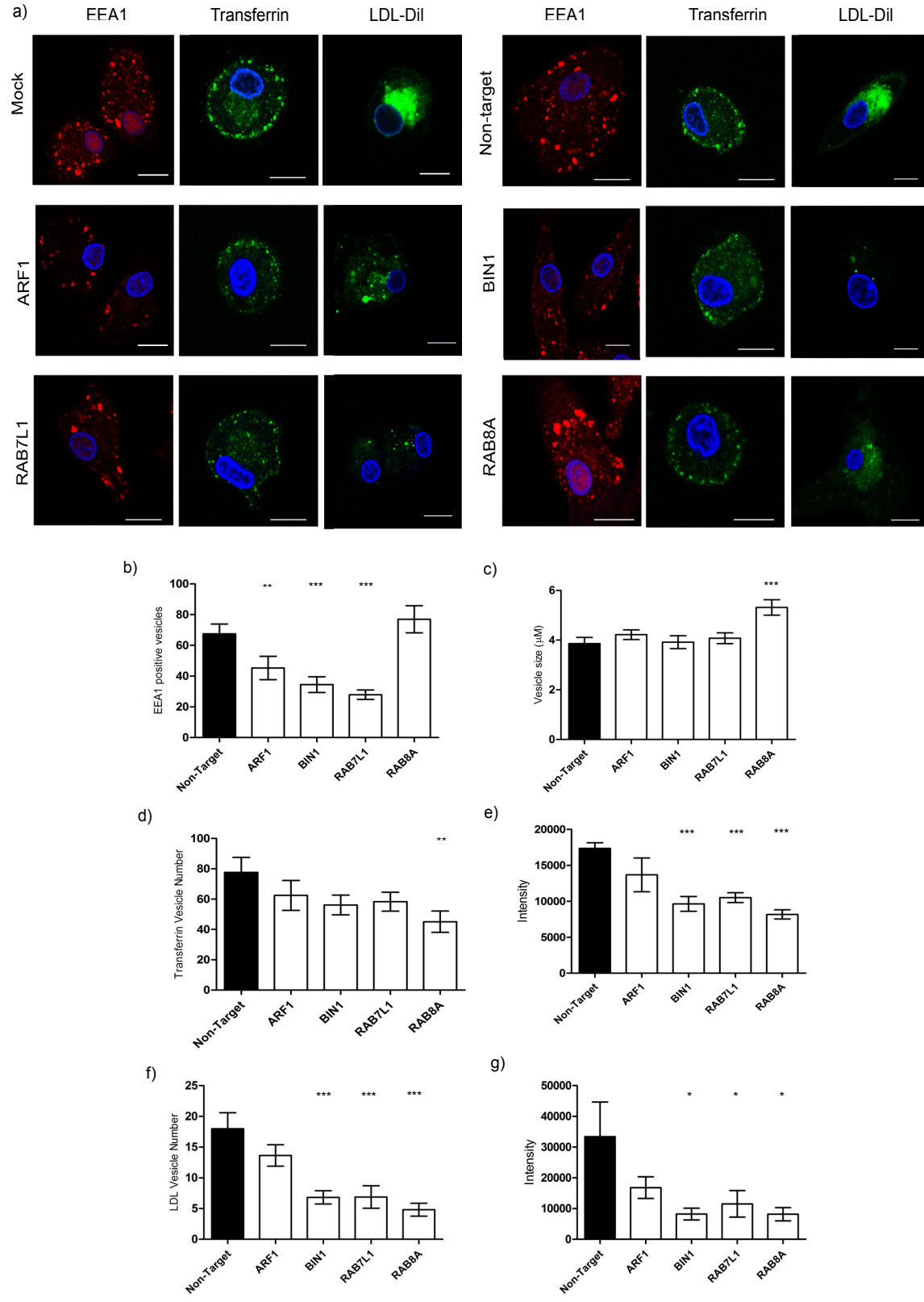


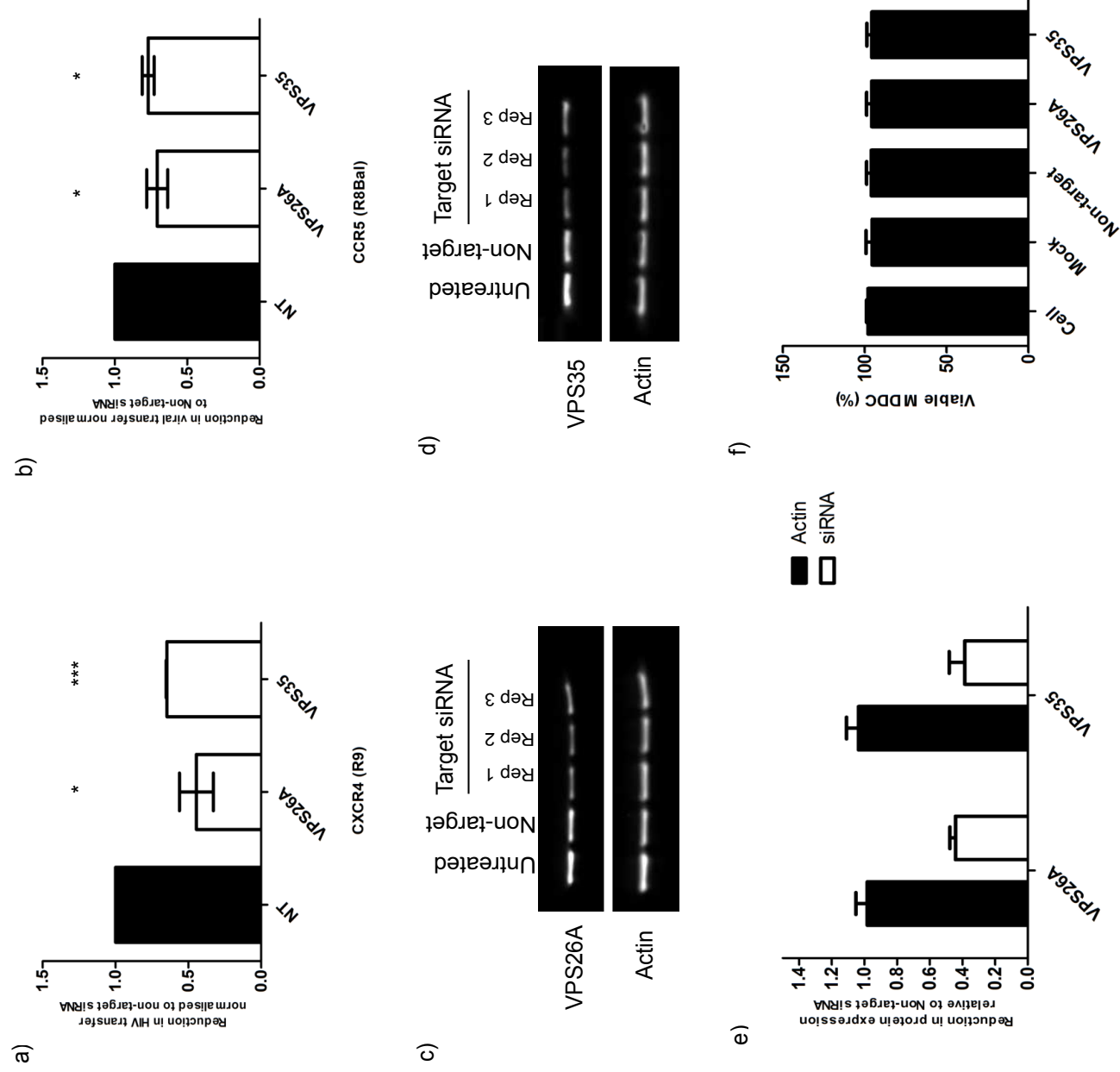












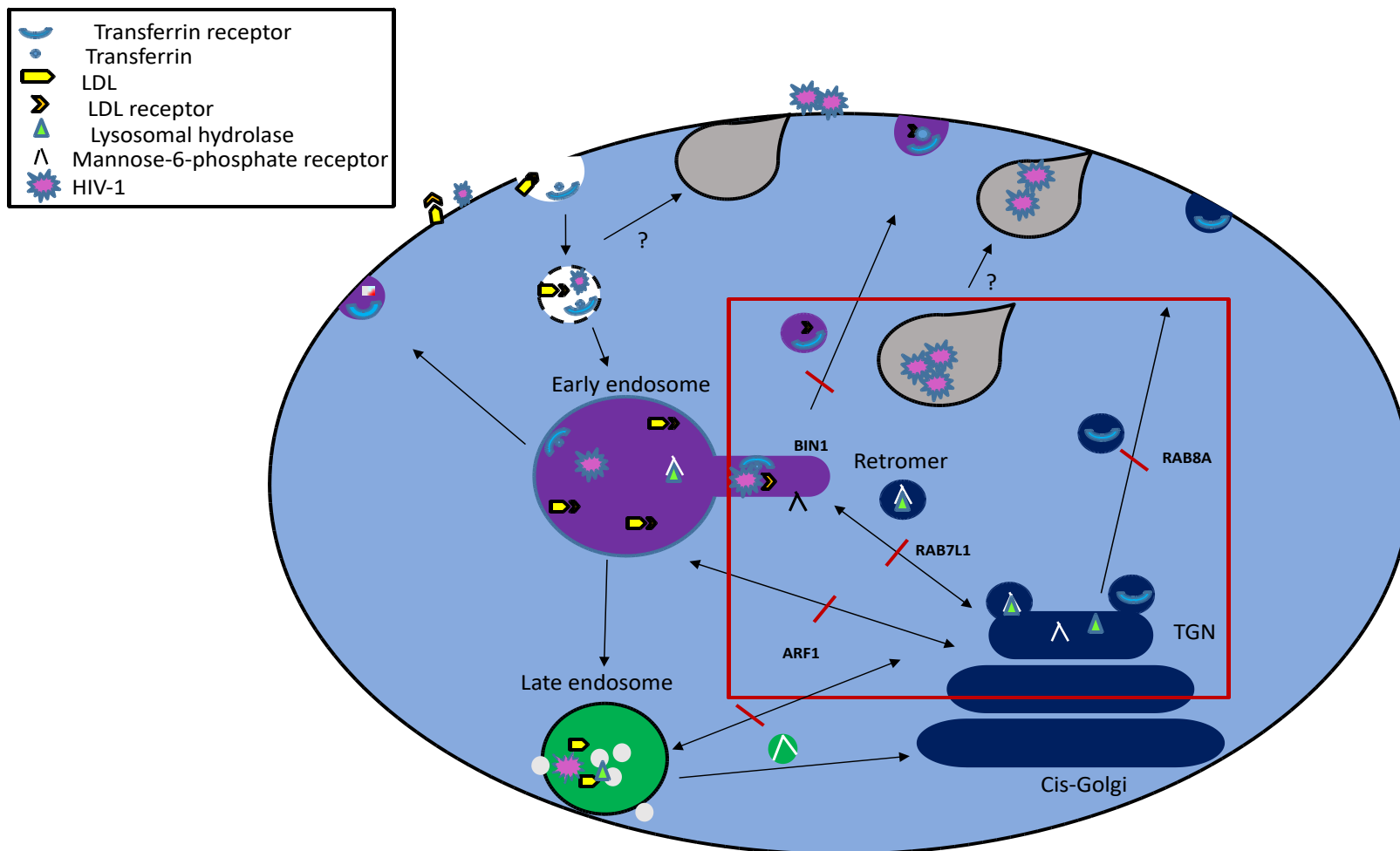


Table 1 Network analysis statistical data

A) Cellular Compartments– Facilitaing Genes			
Nodename	pvalue	qvalue	Dataset size
Cytoplasmic membrane-bound vesicle	1.781257032788977E-5	0.0026505776165784978	7
Cytoplasmic vesicle	2.9736862512104874E-5	0.0022129769452692294	7
Golgi apparatus	7.260275955618954E-5	0.0035995739100488366	7
Clathrin-coated vesicles	1.1280567574068089E-4	0.004193431353399224	4
Trans-Golgi network	1.5015004674908726E-4	0.004464810314555923	4
B) Cellular Compartments– Inhibitory Genes			
Nodename	pvalue	qvalue	Dataset size
Clathrin coat of coated pit	1.98808829214536E-8	2.0278480239444008E-6	4
Clathrin vesicle coat	7.728295638241832E-8	3.9414231596257565E-6	4
Clathrin coat	5.75990999192209E-7	1.9583507854470383E-5	4
Vesicle coat	6.170337924836827E-7	1.5734242778675522E-5	4
Coated pit	1.8932548517714428E-6	3.86216897007019E-5	4
C) Biological Processes – Facilitating Genes			
Nodename	pvalue	qvalue	Dataset size
Vesicle-mediated transport	2.5476631776993885E-7	1.4444208784170076E-4	8
Membrane Organisation	7.045745098037326E-5	0.019777204754682143	6
Endocytosis	1.3846610717114176E-4	0.025832390294862395	5
Vesicle Organisation	2.614347913818171E-4	0.03638479278473983	4
Cellular protein localisation	2.906708321191288E-4	0.03242937937435153	6
D) Biological Processes – Inhibitory Genes			
Nodename	pvalue	qvalue	Dataset size
Vesicle-mediated Transport	2.60964598927977E-5	0.01146851519073222	6
Endocytosis	3.158300588098925E-5	0.006955651219872405	5
Receptor-mediated endocytosis	1.0101208431475834E-4	0.0147729914135587	4
Establishment of protein localisation	1.375341659809643E-4	0.015083655172842936	6
Protein localisation	3.4173910866504216E-4	0.02976299308721586	6